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PHYLOGENETIC ANALYSIS OF FORTY PAKISTANIS *Solanum melongena* ACCESSIONS BY SDS-PAGE

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Abstract: Phylogenetic relationship of 40 accessions of *Solanum melongena* L. collected different geographical areas in Pakistan, revealed the existence of diversity by SDS-PAGE. Intraspecific relationship was constructed by dendrogram with 100% tolerance UPGMA (Unweight Pair-Group Arithmetic Mean). The electrophoretogram of accessions No. (1-19) 018477, 018482 (Fasialabad), 18484 (Sahiwal), whereas accessions from 20-40 from D. I Khan (18504, 18500, 18505, 14466(3), Sahiwal (20344) and Batgram (20509) was unique in protein banding position. As well as 17(24KDa- 20281, 20425, 44663) band was also unique in position. This work demonstrated that accessions have low level of genetic diversity. Cluster analysis conducted separately for each accession, in relation to the genetic status of accession. Largest dendrogram of cluster 1 divided into 6(6a, 6b), 7(7a, 7b), 8(8a, 8b) and 9(9a, 9b) sub clusters including accessions 20425-4745(3). Results indicated that all studied accessions had almost similar protein contents. No relationship was found between genetic divergence and genetic status of sample. The genotypes included in the diverse clusters could be used as promising parents for hybridization in order to obtain a high heterotic response and thus contribute to eggplant breeding.
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Key Word: Phenetic, *Solanum melongena*, SDS-PAGE, Protein, Intraspecific.

INTRODUCTION

Solanum melongena L. belongs to family Solanaceae (Nightshade family). The genus Solanum has 1000 species in the world. *S. melongen* known as eggplant, brinjal, aubergine (French), garden eggs (Nigeria), gauta (Hausa), afufa or anara (Igbo) and igba in Yoruba (Agoreyo et al, 2012). This is a popular vegetable of tropics, subtropics and temperate regions (Lai, 1993). Eggplant is a warm weather crop grown extensively in India, Bangladesh, Pakistan, China, Japan and Philippines (Agnieszka et al, 2007). In Pakistan, area covered by brinjal is 9,044 ha, production 88,1,48 tons and yield 97,466 kg/ ha (Srinivasan 2009). Reported species are 15 from which 12 have medicinal values in Pakistan (Nasir 1985). This specie has therapeutic potential against headache, heart burning and heat of stomach (Edmonds and Chewya, 1997). Eggplant contains nutrients such as dietary fiber, folate, ascorbic acid, vitamin K, niacin, vitamin B6, pantothenic acid, potassium, iron, magnesium, manganese, phosphorus and copper (USDA 2009). Brinjal diet is important for the poorer when other vegetables are in short supply (Srinivasan 2009).

This vegetable is very sensitive to diseases and pests. Some of the important insect pests of brinjal in Pakistan are brinjal fruit borer, *Leucinodes orbonalis* Guenee (Lepidoptera, Pyralidae), brinjal stem borer, *Euzophera perticella* Ragonot (Lepidoptera, Pyralidae), leaf roller, *Eublemma olivacea* (Walker) (Lepidoptera, Noctuidae), beetle, *Epilachna vigintioctopunctata* Fabr. (Coleoptera, Coccinellidae), aphid, *Aphis gossypii* (Homoptera, Aphididae), Whitefly, *Bemisia tabici* (Genn.) (Hemiptera, Alerodydidae), thrips, *Thrips palmi* Karny (Thysanoptera, Thripidae) (FAO 2010) and cotton jassid (CJ), *Amrasca biguttulan biguttula* (Ishida) (Hemiptera, Cicadellidae) (Nagia et al, 1993; Mahmood et al, 2002). Scientists are forced to introduce such species, which have high protein contents and genetically resistant species against diseases (Neilyn et al, 1995).

Taxonomic status of *S. melongena* remained highly controversial in past. Most of the taxonomic information of this genus is morphometry and many issues are unresolved. (Yousaf et al, 2006). Phenotypically this specie is highly polymorphic

ranging from wild, weedy to semi or fully-cultivated forms at inter and intra specific landraces. Morphological characters such as origin and arrangement of spines, presence of hairs on petiole and stem, flower and fruit colour, length of filaments and style showed polymorphism (Lester and Hassan, 1991; Karihaloo et al, 2002). They also vary in fruit color, shape and size (Akanitapichat et al, 2010; Chinedu et al, 2011). *S. melongena* is small, white in colour, having three varieties that are round or oval and oblong shape; yellow and red in colour, when they are ripe and overripe respectively. Brinjal varieties KB9, Pusa Purple Long, KP10, BB1(Gaiwad et al, 1991), A 300 (Mistasa), Abar, Parat, EG 2003, Mara and Acc 612 (Suiza 1997) are tolerant and resistant to insect pests.

Genetic diversity in species of *S. melongena* is the best source of breeders and hybridized specie resistive against susceptibility (Lantican, 1993). Exclusive breeders can be produced by infusion of hybridized and genetically diverse specie of *S. melongena* (Welsh, 1981). Molecular markers have enormous potential to explore genetic diversity due to polymorphisms. These are useful tools for breeding genotype identification and the determination of genome organization and evolution in plants (Clain et al, 2004). SDS-PAGE is fairly simple, cheaper and rapid to perform (Leisner et al, 2001). SDS-PAGE is effective for phylogenetic relationships at inter and intra specific to resolve systematic (Karihaloo et al, 2002; Edmonds and Glidewell, 1977).and taxonomic confusions at generic, species level (Harborne and Turner, 1984), sub-species and infra sub-species level (Jackman, 1985; Kersters, 1985; Khalifa et al, 1998). In modern years, complex taxonomic problems are resolved by SDS-PAGE of total seed protein profile (Quicke, 1993; Abou-El-Enain, 1995). Protein richest specie can be evaluated by this tool.

Brinjal accessions exhibited high level of resistance against diseases caused by insect pests (Khan et al, 2015). In this research work forty accessions were collected from different geographical areas of Pakistan. The results of this study not only provide the information for breeders but also helpful for taxonomists to clarify the phylogenetic relationship amongst the vast genetic diversity. Seed protein profiles of 40 accessions belonging to *S. melongena* were resolved by SDS-PAGE. Intraspecific relationship was estimated by dendrogram based on UPGMA revealed the accession status of *S. melongena.*

The objective of the present work is to resolve the existing intraspecific taxonomic relation and protein richest accessions of *S. melongena* by using SDS-PAGE.

MATERIALS AND METHODS

Plant material and seed preparation

The study was conducted in Molecular Taxonomy lab, Department of Botany, Lahore College for Women University Jail road Lahore Pakistan. The experiment was designed to evaluate taxonomic confusion among the genetically diverse accessions of *Solanum melongena* by proteins markers. The proposed study was comprised of seed protein analysis of 40 accessions of *S. melongena* by SDS-PAGE. The seed materials were taken from Gene bank of Agri biotechnology and genetic resources (IABGR) institute, National Agricultural Research Center (NARC) Islamabad and Katholieke University Nijmegen, Hortus Botanicus Netherlands.

Seed protein analysis by SDS-PAGE

a) Seed storage Protein extraction:

The meal from a composite sample of 5 to 12 seeds for each accession was analyzed. Each sample was prepared by pounding cotyledons to flour by pastel and mortar; the total proteins were extracted. In Eppendrof tubes 10 mg seed powder was poured in 400 µl urea extraction buffer and shake well with glass rod. Extraction buffer contained 0.05 M Tris-HCl (pH 8.0), 0.2 % SDS, 5 M urea and 1% β-marcaptathenol. Seed flour was thoroughly mixed with buffer by vortexing. The extracts were centrifuged at 15,000 rpm for 5 minutes. The crude proteins were recovered as supernatant, transferred into new 1.5 ml Eppendr of tube and stored at -20°C until the time of analysis (Sammour et al, 1994). Proteins in the supernatants were quantified using Bio-Rad DC protein assay (USA) and on the gel, Fermentas (80 kDa (kilodalton) - 21 kDa) were used as marker.

***b) Electrophoresis*:**

One-dimensional SDS-PAGE was performed according to modified Laemmli method (1970) to carry protein analysis by vertical slab gel in discontinuous buffer system. The resolving gel concentration was 12% or 15 % and thickness of slab gel was varied from 0.75-1.5 mm. The concentration of resolving gel was depending on the nature and size of protein. But in this experimental work 12 % was used. The stacking gel was loaded upon resolving gel for 40 min until polymerization started. The 12 µl protein buffer was loaded on 12% SDS-PAGE. Electrophoresis was performed in the Protean II electrophoresis cell (Bio-Rad USA) at 100 V until the bromophenol dye (BDH laboratory Supplies Poole, England) reached the bottom of the gel.

***c) Staining and destaining*:**

The gels were stained in the staining solution containing 44% methanol, 6% acetic acid, 500ml distilled water and 2.25g of coomassie brilliant blue (Sigma Aldrich Chemie, Germany) for 45mins.

Destaining solution contained 20% methanol, 5% acetic acid and 750 ml of distilled water until the background color disappeared and protein bands were clearly visible (Yousaf et al, 2006). Dry the destained gel in drying processor at 60-70°C for 1hour (Yousaf et al, 2006).

d) Data Analysis:

Dissociated polypeptide weight can be determined by plotting standard curve of log 10 molecular weight of standard polypeptide against the calculated relative mobility (Rf) value (formula 1) of each protein.

R_F **= Distance migrated by protein (cm) / Distance migrated by marker (cm)**

Standard curve was used to determine the log molecular weight. Unknown protein value was determined by antilog of this number. The protein ladder consisted of Albumin, from Bovine plasma (66 KDa), Egg oval albumin (45 kDa), pepsin from porcine, stomach mucosa (34.7 KDa), Trypsinogen from Bovine Pancreas (24kDa) and B-Lactoglubulins (18.4 kDa). These protein markers were available in Sigma Chemical Company, USA (Table 1).

e) Statistical Data Analysis:

The estimation of phylogenetic relatedness within and among the samples was based on 18 reproducibly scored bands identified in the zones of highest variation of the protein profiles (ranged from 80 to 21 KDa). The genetic diversity among the accessions was evaluated by using cluster analysis. Protein bands were scored depending on their presence (1) or absence (0). Principal components of data were used as input variables for cluster analysis using Unweighted Pair Group Method with Arithmetic Mean (UPGMA). Using the 'Graphics'option, the computed UPGAM data were used to construct a dendrogram.

RESULTS AND DISCUSSION

In this experiment, degree of relationship and phylogenetic variations of *S. melongena* accessions have been explored by 12 % SDS-PAGE. Same concentration was used by Yousaf et al, (2006). The advantages of SDS-PAGE is 1) The universal distribution of the proteins so that there is no theoretical limits to using the electrophoretic markers, 2) the electrophoretic markers are less effective to the environmental fluctuations, 3) they are too proximity to the primary genetic information (third hand copy of DNA), 4) the discriminatory power of electrophoresis is usually very high and distinction between genotype can often be achieved with less effort and with the analysis of fewer individuals than the morphologically based system (Sammour 1991), 5) favor identification of lower order taxa (Yousaf et al, 2006) 6) the operating costs are relatively low and showed high resolution (Holmes et al, 1990)

Electrophoretic analysis of seed storage proteins used for identification of cultivar, breeder, plant varieties and accessions. Seed protein profiles are known for stability, uniformity and additive nature (Rao et al, 1990; Stegemann et al, 1992; Yupsanis *et. al.*, 1992). Electrophoresis is also used to check the pedigree (Thanh et al, 2006) and systematics of sub-specie level (Quake, 1994). Kamel (1996) had studied some relationship between certain taxa, species and tribes of certain families. Abou El-Enain (1995) had determined the taxonomic and phylogenetic relationship of *S. melongena* seed protein by SDS-PAGE.

Diffusion of crop from its primary center of diversity to secondary regions (Behera et al, 2006), where primitive cultivars and weed (Daunay 2008) caused the diversification and evolution (Prohens et al, 2005) of crop due to micro-evolutive factors like mutation, selection (natural and artificial), genetic drift, gene flow and recombination Muñoz-Falcon et al, (2008) worked on Spanish striped and non-striped egg plant accessions and their results based on AFLP.

In this work 18 bands were observed (Fig 2, 3). The intensity of bands is represented by three different colors, number, molecular weight, width and presence/absence of bands. Protein profiles were categorized into major (dark colour) and minor bands (light colour). Nunome et al, (2001) proved that *Solanum melongena* accessions have minor variation and low frequency of polymorphism.

Electrophoretogram of *S. melongena* accessions were used to evaluate the genetic variability. The protein profile of forty accession of *S. melongena* ranged between 80-21 KDa. Protein profile of 1-19 accessions indicated that 18 (21 KDa) and 7 (49 KDa) band showed exclusive similarity in banding position (Fig 2). In electrophoretogram of all 40 accessions (Fig 2, 3) bands 1 (80 KDa), 5 (52 KDa), 8 (44 KDa), 15 (27 KDa), and 16 (26 KDa) was highly constant, uniform and frequent might be the species specific and remained same generation after generation and not effected by environmental stresses. Karihaloo et al, (2002) concluded that most of the accessions had identical band patterns, supporting interbreeding complex with limited genetic differentiation. So these bands may be an important marker for the identification of *S. melongena*. Minor bands contributed to determine the variation in this experiment. It might be possible to divide the genotype into different groups and cultivars.

The approximate number of deeply stained bands was 36 in 40 accessions belong to Faisalabad, Sahiwal, Punch, D.I Khan, Bahawalpur,

T.T Singh, Swat and Vehari. But only two accessions were lightly stained *i.e.* 18489 (Acc.) and 18494 (Acc.) from Punch and D.I Khan respectively.

To divide the accessions into distinct groups, variation in the staining intensities of minor bands were not enough. Syed and Isa, (1998) worked on the accessions of different regions of world to evaluate the similarity and differences among accessions. Results of electrophoretic banding pattern showed the great deal of genetic variation within and between groups in terms of number, size, position, staining intensities and presence or absence of bands in profile of round and elongated fruits (Karihalo and Rai 1995).

Protein profile 2 and 7 (1-40 accessions) showed specific protein banding pattern of *S. melongena*, which indicated the soil and environmental impact on the accessions. The band 2 (70 Kda) of Mandi Bahiddin (18481), Sahiwal (18485), D.I Khan (18499, 18504), Swat (20344) and Batgram (20509) and band 7 from Faisalabad (18477, 18482), D.I Khan (18496, 18499, 18500, 18505) and Bahawalpur (4466-3) indicated that their soil has similar characteristics. Anu and Peter, (2003) analyzed seed protein of 29 accessions of *Capsicum annum* L. by polyacrylamide gel electrophoresis. They observed distinct genotypic bands but certain bands were shared by several genotypes.

The electrophoretogram of (1-19 accessions) (Fig2) band 13(36Kda) was considered accession (018484) specific band called minor band due to light in colour, whereas accession (20-40) (Fig 3), it was categorized as major bands. But this band was not providing enough data to identify and divide the species into groups. The results were verified by UPGMA. Ono (1996) observed 7 (BP1, BP2, BP3, BP4, BP5, BP7, BP8). polymorphic bands due to sufficient genetic diversity among local collections of *S. melongena* by electrophoresis. Singh et al, (2005) disagreed with Ono. Diversity and their relationships of the cultivated species facilitate the establishment of conservation strategies, genetic resources in breeding programmes and crop evolution (Maria et al, 2012).

Cluster analysis help to characterize the accessions into several distinct and significant groups (sub-species, botanical or variety group, cultivar and population). So accessions collected from different areas are intermixed showed no genetic barrier (Table 3).

Cluster 9 was the largest cluster comprising most of the accessions. It was divided into two main sub clusters 9a and 9b. Sub cluster 9a contained seven accessions 18497, 18478, 20537, 20480, 18491, 18498, 18489 while 9b comprised 11 accessions 20295, 19868, 4745(3), 20507, 20281, A-58, 4792(3), XIANGZUE-6, 20257, White-Egg, MK-95. In subgroup 9b accessions comprised genetic variability up to 2-26%, collected from T.T. Singh, Bahawalpur, Raheem Yar Khan and Mansehra (Fig 4)

Cluster 8 also divided into two sub clusters 8a and 8b. Subgroups 8a included three accessions 4466(3), 18505, 18496 collected from (Bahawalpur, D.I. Khan) and in 8b two accessions (188500, 18482) from D.I. Khan, Faisalabad. (Fig 3) This shown genetic variability up to 27%. Cluster 7 divided into two sub clusters 7a and 7b showed genomic variation 2-32 % (Fig 4). Sub cluster 7a containing 20452, 20344 collected from Vehari, Swat. Sub clusters 7b containing 18504, 18485 from D.I. Khan, Sahiwal showed more similarity than 7a accessions.

Cluster 5 divided into two subgroups 5a (18476, 18475) and 5b (18495, 18494) whereas cluster 6 containing three sub clusters (18473, 18479, 18474) showed geographical areas were not contribute to genetic variability in accessions. Cluster 4 contained 18502, 18484 and cluster 3 contained only one accession 18477. Cluster 2 comprised 18499 and 18481 accessions, Cluster1 contained one accession 20509 (Fig 4). Tümbilen et al, (2011) studied the genetic variability of 67 Turkish eggplant accessions with 30 morphological traits. Dendrogram of high genetic similarity in related Solanum species ranged from 0.30 to 0.95 whereas 0.68 to 0.95 indicating low genetic diversity. Behera et al, (2006) worked on closely related species of Solanum clustered along *S. melongena* accessions, being crossable with cultivated species, constitute important sources of genes that can be introgressed by backcross breeding. Molecular markers can be employed to identify the hybrids and also to monitor introgression of useful genes.

On geographical map, Pakistan is closely related to Indian subcontinent (Tsao and Lo, 2006). But India is very diverse in eggplant germplasm in different regions substitutes the high level of genetic variability as compare to Pakistan which showed minute or negligible variation (Whalen, 1984). So the similarities in the banding pattern of accessions were common parents in their pedigree but patterns were slightly different if the accessions were multiplied from the small sample size (Gardiner and Forde, 1987).

Table 1: Protein standards with molecular weight, Log size and their Rf Value

Standard protein	Molecular weight (Kda)	Log Size	Rf Values (cm)
Albumin, Bovine Plasma	66	4.82	0.23
Albumin, Egg Oval-bumin	45	4.65	0.35
Pepsin Porcine Stomach Mucosa	34.7	4.54	0.48
Trypsinogen, Bovine Pancreas	24	4.38	0.52
B-Lactoglobulin, Bovine Milk	18.4	4.26	0.62
Marker (dye at the end)			0.85

Table 2: List of forty accessions of *Solanum melongena* used in this study

S. No.	Acc. No.	Geographical area	Geographical location
1	018473	Faisalabad	31° 25' 0" North, 73° 5' 0" East
2	018474	Faisalabad	31° 25' 0" North, 73° 5' 0" East
3	018475	Faisalabad	31° 25' 0" North, 73° 5' 0" East
4	018476	Faisalabad	31° 25' 0" North, 73° 5' 0" East
5	018477	Faisalabad	31° 25' 0" North, 73° 5' 0" East
6	018478	Faisalabad	31° 25' 0" North, 73° 5' 0" East
7	018479	Faisalabad	31° 25' 0" North, 73° 5' 0" East
8	018481	Mandi Bahauddin	32° 35' 0" North, 73° 30' 0" East
9	018482	Faisalabad	31° 25' 0" North, 73° 5' 0" East
10	018484	Sahiwal	30° 40' 0" North, 73° 6' 0" East
11	018485	Sahiwal	30° 40' 0" North, 73° 6' 0" East
12	018489	Punch	3° 46' 0" North, 74° 6' 0"East
13	018491	D.I. Khan	31° 49' 58" North, 70° 54' 9" East
14	018494	D.I. Khan	31° 49' 58" North, 70° 54' 9" East
15	018495	D.I. Khan	31° 49' 58" North, 70° 54' 9" East
16	018496	D.I. Khan	31° 49' 58" North, 70° 54' 9" East
17	018497	D.I. Khan	31° 49' 58" North, 70° 54' 9" East
18	018498	D.I. Khan	31° 49' 58" North, 70° 54' 9" East
19	018499	D.I. Khan	31° 49' 58" North, 70° 54' 9" East
20	018500	D.I. Khan	31° 49' 58" North, 70° 54' 9" East
21	018502	D.I. Khan	31° 49' 58" North, 70° 54' 9" East
22	018504	D.I. Khan	31° 49' 58" North, 70° 54' 9" East
23	018505	D.I. Khan	31° 49' 58" North, 70° 54' 9" East
24	019868	Bahawalpur	29° 24' 0" North, 71° 41' 0" East
25	020257	Bahawalpur	29° 24' 0" North, 71° 41' 0" East
26	020281	Bahawalpur	29° 24' 0" North, 71° 41' 0" East
27	020295	T.T. Singh	30° 58 0 N0rth, 72 29 0 East
28	020344	Swat	35° 22' 42" North, 72° 10' 47" East
29	020452	Vehari	30° 1' 30" North, 72° 42' 10" East
30	020480	Kohat	33° 35' 13" North, 71° 26' 32"East
31	020507	Mansehra	34° 20' 0" North, 73° 12' 0" East
32	020509	Batgram	34° 56' 58" North, 72° 48' 30" East
33	020537	Kohat	33° 35' 13" North, 71° 26' 32" East
34	4466(3)	Bahawalpur	29° 24' 0" North, 71° 41' 0" East
35	4745(3)	R.Yar Khan	28° 23' 2" North, 70° 16' 46" East
36	4792(3)	R.Yar Khan	28° 23' 2" North, 70° 16' 46" East
37	A-58	Bahawalpur	29° 24' 0" North, 71° 41' 0" East
38	MK-95	Bahawalpur	29° 24' 0" North, 71° 41' 0" East
39	White-Egg	Bahawalpur	29° 24' 0" North, 71° 41' 0" East
40	XISANGZUE-6	Bahawalpur	29° 24' 0" North, 71° 41' 0" East

Table 3: Details of *S. melongena* accessions used for protein gel electrophoresis.

Cluster	Acc. No.	Geographical Position
Cluster 1	20509	Batgram
Cluster 2	18499, 18481	D. I Khan, Mandibahauddin
Cluster 3	18477	Faisalabad
Cluster 4	18502, 18484	Sahiwal
Cluster 5a	18476, 18475	Faisalabad
Cluster 5b	18495, 18494	D. I Khan
Cluster 6	18473, 18479, 18474	Faisalabad
Cluster 7a	20452, 20344	Vehari, Sawat
Cluster 7b	18504, 18485	D. I Khan, Sahiwal
Cluster 8a	4466(3), 18505, 18496	Bahawalpur, D. I Khan
Cluster 8b	18500, 18482	D. I Khan, Faisalabad
Cluster 9a	18497, 18478, 20537, 20480, 18491, 18498, 18489	Faisalabad, D. I Khan, Kohat, Punch
Cluster 9b	20295, 18868, 4745(3), 20507, 20281, A-58, 4792(3), XIANGZUE-6, White-egg, MK-95	T. T Singh, Bahawalpur, Rahim Yar Khan, Mansehra

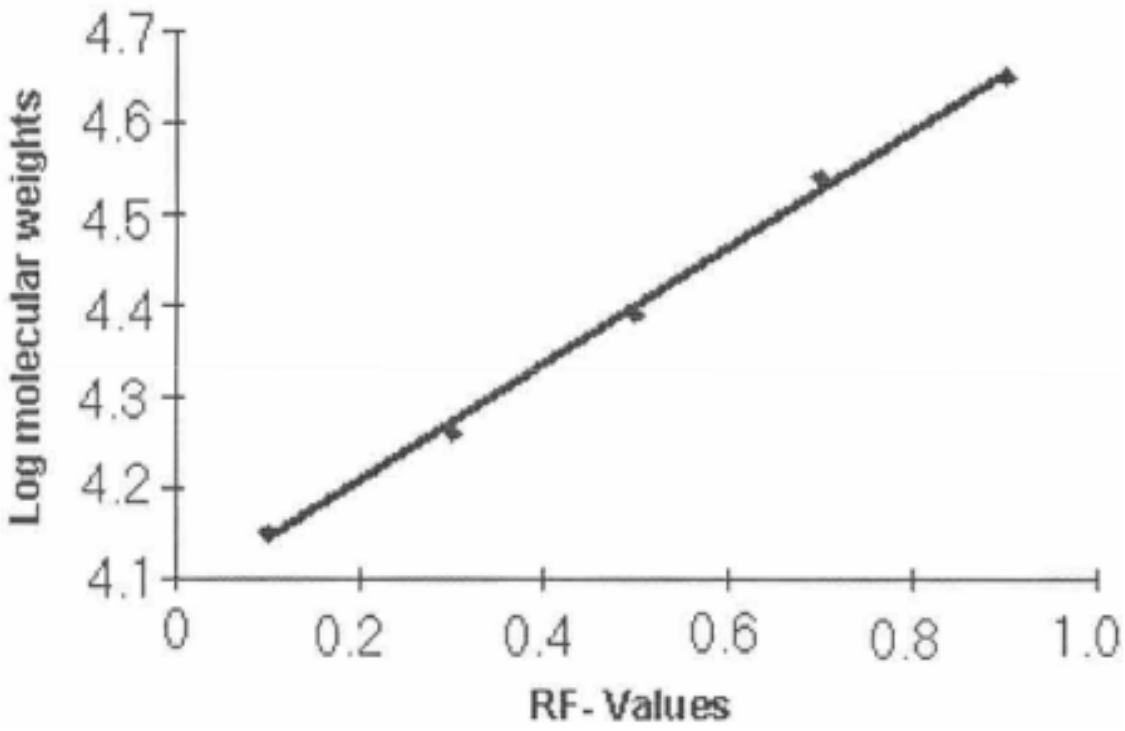

Fig 1: Standard curve of Protein analysis

Calculation:
66Kda = 66000
Log 66000 = 4.82 log size

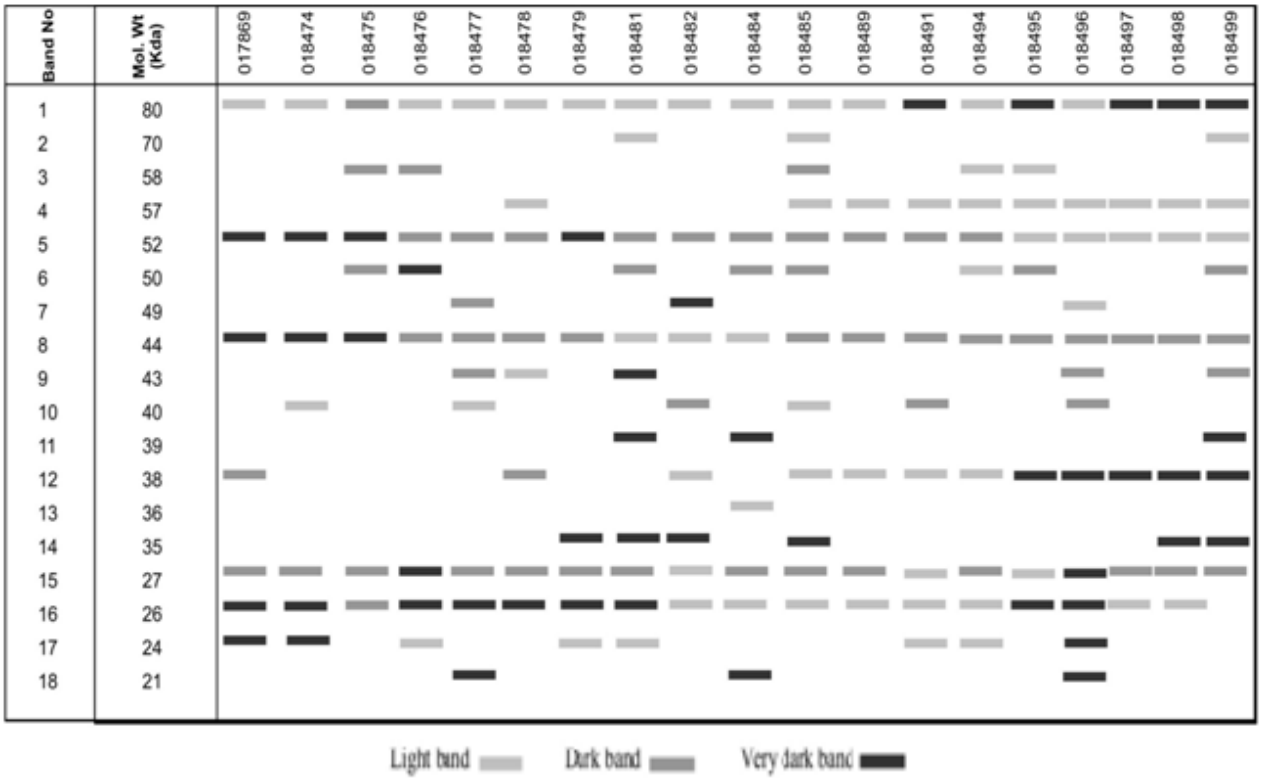

Fig 2: Electrophoretogram of 1-19 accessions of *Solanum melongena* based on 12 % acrylamide gel.

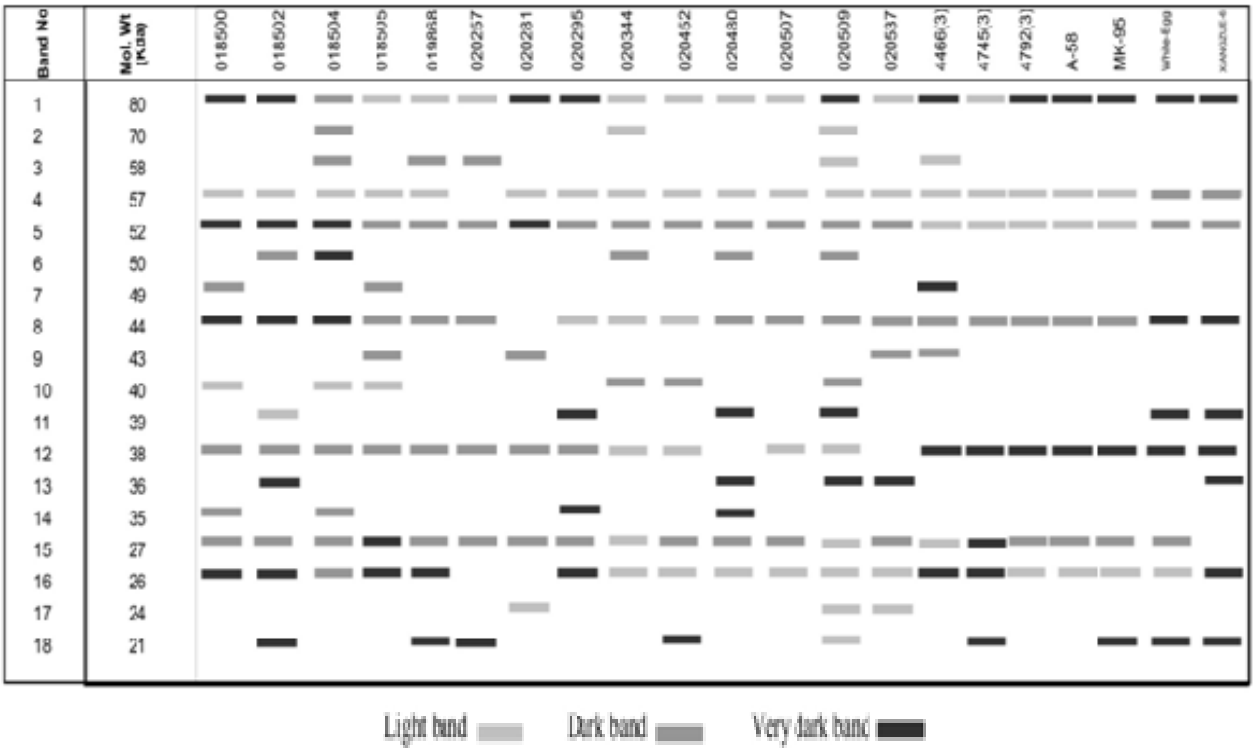

Fig 3: Electrophoretogram of 20-40 accessions of *Solanum melongena* based on 12 % acrylamide gel.

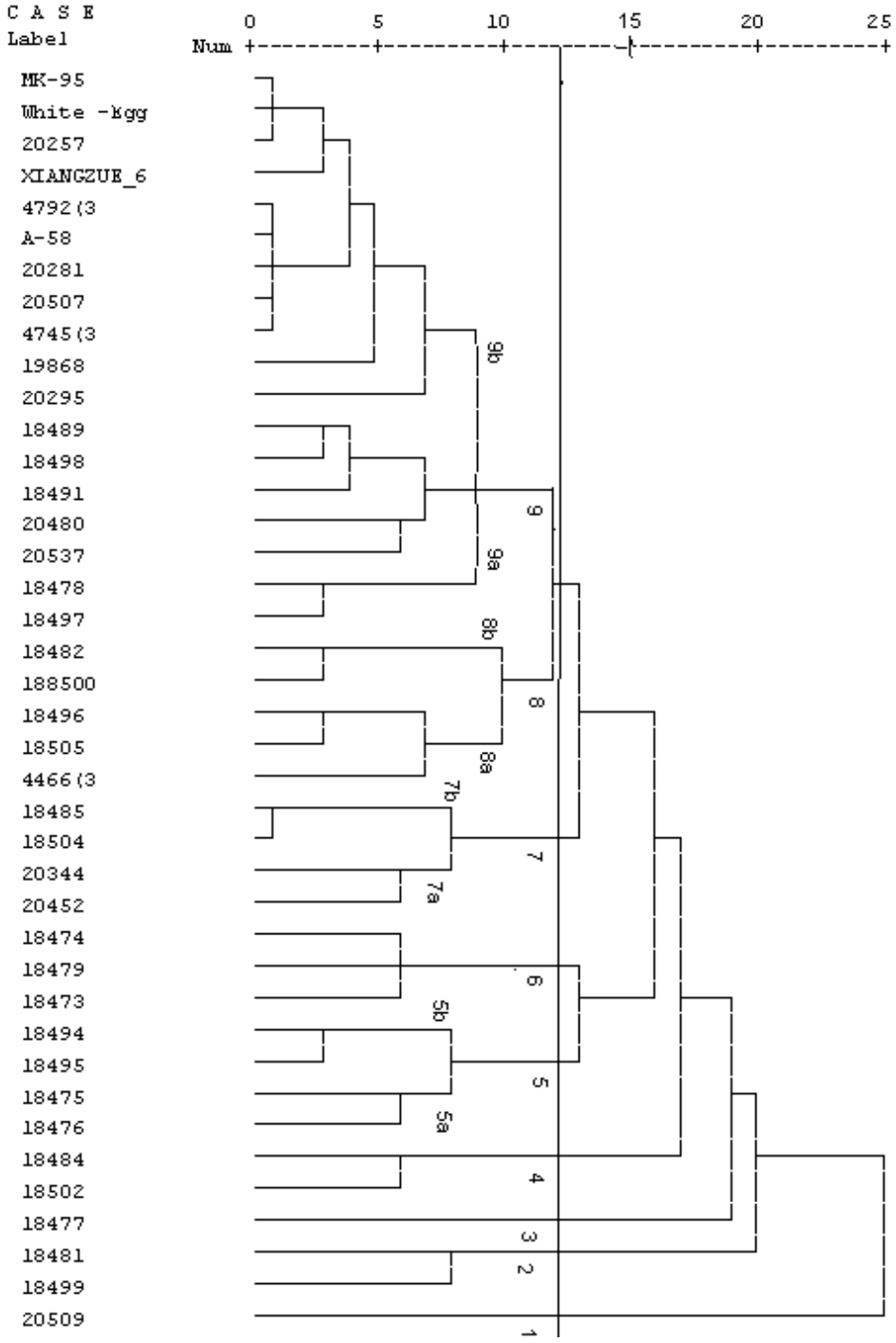

Figure 4: Cluster analysis dendrogram of 40 accessions of *Solanum melongena* based on seed protein gel electrophoresis (0.30-0.95%)

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CONCLUSION

The conclusion of this experimental result was to explore the major as well minor diversity among the accessions of *S. melongena* by SDS-PAGE. According to results it has proved that geographically distributed accessions of *S. melongena* have low level of polymorphism, which was negligible. So this low level cannot be used to eliminate the taxa. The study of the diversity of geographically distant centers of diversity, where eggplant was introduced through different routes provided information for understanding the structure of variation in eggplant, as well as for the conservation of genetic resources and breeding of this crop.

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